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Thanks.
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Tumor-selective Prodrug Activation by Fusion Protein-mediated Catalysis

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ABSTRACT

A two component system, consisting of a fusion protein and an appropriate prodrug, suited to perform selective tumor therapy *in vivo* is presented. The fusion protein, due to its humanized carcinoembryonic antigen-specific variable region, specifically binds to carcinoembryonic antigen-expressing tumors and has an enzymatic activity comparable to that of human β-glucuronidase. The prodrug is a nontoxic glucuronide-spacer derivative of doxorubicin decomposing to doxorubicin by enzymatic deglucuronidation.

***In vivo* studies in nude mice bearing human carcinoembryonic antigen-expressing tumor xenografts revealed that 7 days after injection of 20 mg/kg fusion protein a high specificity ratio (>100:1) was obtained between tumor and plasma or tumor and normal tissues. Injection of 250 mg/kg of prodrug at day 7 resulted in tumor therapeutic effects superior to those of conventional chemotherapy without any detectable toxicity. These superior therapeutic effects which were observed using established human tumor xenografts can be explained by the approximately 4–12-fold higher doxorubicin concentrations found in tumors of mice treated with fusion protein and prodrug than in those treated with the maximal tolerable dose of drug alone.**

The nondetectable toxicity in the animals treated with fusion protein and prodrug is probably caused by up to 5-fold lower drug concentrations in normal tissues compared to the animals treated with doxorubicin. Thus, a more tumor-selective therapy, resulting in stronger therapeutic effects and reduced toxicity seems to be possible by the appropriate use of the humanized nontoxic fusion protein and the nontoxic prodrug.

INTRODUCTION

The major limitations of conventional chemotherapy are its lack of tumor selectivity resulting in high toxicity as well as generation of multidrug-resistant tumor cells under the influence of long-term treatment with insufficient drug concentrations at the tumor site (for review see Ref. 1). To overcome these problems of toxicity and multidrug resistance several groups tried to develop antibody-enzyme conjugates which ideally shall activate nontoxic prodrugs to toxic drugs in high concentrations at the tumor site only (for review see Ref. 2).

To get these site-specific activation systems working at least two or preferably three steps are needed (3). The first step includes the injection of a tumor-selective antibody-enzyme conjugate into the tumor-bearing individual. After an appropriate localization phase of 1 or 2 days a second antibody directed to the enzyme is injected in order to clear the antibody-enzyme conjugate from the plasma. Thereafter a nontoxic prodrug which can be cleaved to a cytotoxic drug by the enzyme moiety of the antibody-enzyme conjugate localized at the tumor is injected.

The whole procedure as shown by studies in nude mice and by preliminary clinical trials generates superior therapeutic effects in comparison to conventional chemotherapy (3, 4). Nevertheless, the high immunogenicity of the antibody-enzyme conjugates, which consist of murine monoclonal antibodies chemically linked to xenogenic enzymes, does not allow repetitive application of the conjugate leading to a limitation of the therapy. Furthermore, the insufficient clearance of the antibody-enzyme conjugates and the inappropriate plasma stability of the prodrug result in high concentrations of drug in the plasma. This deficiency causes a significant toxicity to nontumorigenic tissues (4).

To reduce the problem of immunogenicity our group has generated a fusion protein consisting of a humanized CEA[2]-specific binding region and human β-glucuronidase using recombinant DNA technology (5). Under native conditions, the fusion protein has an apparent molecular weight of >250,000 and is composed of 2 heavy and 2 light chains. The enzyme moiety of the fusion protein is located at the carboxy-terminal part of the heavy chains of the fusion protein. Further characteristics of the fusion protein with respect to its specificity and avidity as well as enzymological and protein chemical properties have already been described in detail (5).

In the present study we present information concerning the pharmacokinetics, tumor and tissue distribution, and metabolism of the fusion protein after i.v. injection in human tumor-bearing nude mice. Furthermore, we present data on the pharmacokinetics, organ distribution, stability, and toxicity of an appropriate glucuronyl-spacer-doxorubicin prodrug, the synthesis (6) and *in vitro* cleavability by human β-glucuronidase of which was described before (6, 7).

In addition, data concerning the drug concentrations in tumor tissue and normal organs after therapy with fusion protein and prodrug are shown and compared with values received using chemotherapy with doxorubicin. Finally, data concerning the therapeutic efficiency of the concept of FMPA on the growth of established human tumor xenografts in nude mice are reported.

MATERIALS AND METHODS

Animals. Female nude mice (CD-1-*nu/nu*) were obtained from Hagemann (Salzfeld, Germany) with a weight of about 18 g. Female CD rats were obtained from Charles River Wiga (Salzfeld, Germany). Monkeys were obtained from the breeding colony of Behringwerke (Marburg, Germany). The different species were housed in appropriate cages and fed with tap water and standard diet *ad libitum*.

Tumor Implantation. Implantation was performed by s.c. injection of 0.2 ml of a cell suspension containing 2×10^6 tumor cells in the right dorsal region of nude mice.

Purification and Analysis of Fusion Protein. The molecular characteristics of the fusion protein used were described in detail (5). Briefly, the recombinant protein has under native conditions a molecular weight of >250,000 and consists of two heavy chains and two light chains having under denaturing conditions a molecular weight of 100,000 or 25,000, respectively. The fusion protein binds with high avidity to CEA via its humanized variable region derived from the mouse MAb BW 431/26 (8) and has β-glucuronidase activity mediated by human β-glucuronidase which represents the carboxy-terminal part of the heavy chains of the fusion protein.

Serum-free transfectoma supernatants containing the fusion protein were purified using antiidiotype affinity chromatography as described (5). The immunoreactivity of the purified fusion protein was determined using a quantitative binding assay in antigen excess as described in detail (9). Briefly, increasing amounts of formalin-fixed CEA-expressing human HT 29 colon

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[2] The abbreviations used are: CEA, carcinoembryonic antigen; FMPA, fusion protein mediated prodrug activation; MAb, monoclonal antibody; BSA, bovine serum albumin; OFAT, organ fusion protein activity test; OEAT, organ enzyme activity test; FU, fluorogenic units; HPLC, high pressure liquid chromatography; MTD, maximal tolerable dose; ADEPT, antibody-directed enzyme prodrug therapy; %T/C, percentage of treated *versus* control.

carcinoma cells were added to 50 ng of purified fusion protein. After overnight incubation at 4°C the antigen was spun down and the amount of unbound fusion protein in the supernatant was determined using a quantitative fluorometric assay for β-glucuronidase (10). The fraction of unbound fusion protein detectable in the supernatant in antigen excess represents the inactive fraction of the purified fusion protein. Immunoreactivity (IR) was determined as

$$IR = 100\% - \frac{100\% \times \text{fusion protein concentration in supernatant at antigen excess}}{\text{Input fusion protein concentration}}$$

For *in vivo* studies highly purified fusion protein isolated using antiidiotype affinity chromatography (5) was applied. Its immunoreactivity was >90% as determined using the quantitative binding assay in antigen excess as described above.

The specific enzyme activity of the fusion protein was determined for two fusion protein concentrations with 4-methylumbelliferyl-β-D-glucuronide as substrate. Data processing was performed using the computer program Grafit (Erithacus Software Ltd., Staines, United Kingdom). Specific activity was determined to be 12 μmol/mg × min at 37°C at pH 4.5.

Preparation of Organs for Fusion Protein or Human β-Glucuronidase Quantification. Animals were sacrificed at the time intervals indicated; organs were removed and weighed. After addition of 2 ml 1% BSA in phosphate-buffered saline, pH 7.2, organs were homogenized using a 5-ml Potter Elvehjem homogenizer (Braun, Germany). Homogenates were adjusted to pH 4.2 and centrifuged at 16,000 × *g* for 30 min. The clear supernatants were neutralized using 0.1 N NaOH and evaluated for functionally active fusion protein content in the OFAT or for human β-glucuronidase in the OEAT.

OFAT. The amounts of intact fusion protein extracted from animal tissues and plasma were determined using the OFAT. Goat anti-human κ antibody (Southern Biotechnology Associates) was attached to individual wells of round-bottomed microtiter plates (Nunc, Germany). After blocking of nonspecific sites with 1% casein, pH 7.2, 50 μl of fusion protein containing sample were added and incubated for 30 min at room temperature. After extensive washing of the plates, 50 μl of a 2.5 mM 4-methylumbelliferyl-β-D-glucuronide solution in 200 mM sodium acetate buffer, pH 5.0, were added and incubated for 24 h at 37°C. The reaction was stopped using 100 μl of 0.2 M glycine + 0.2% sodium dodecyl sulfate, pH 11.1. FU were translated to fusion protein concentrations based on appropriate standard dilution curves determined with purified fusion protein (5).

OEAT. The amounts of enzymatically active human β-glucuronidase (fusion protein associated or free) extracted from animal tissues and plasma were determined using the OEAT. MAb BW 2118/179 selective for native recombinant human β-glucuronidase was attached to individual round-bottomed microtiter plates (solid phase enzyme-linked immunosorbent assays, immunohistochemical staining as well as Western blotting analysis revealed that the MAb did neither bind to murine, hamster, or *Escherichia coli* β-glucuronidase). The plates were processed as described for the OFAT. The FU measured in the OFAT subtracted from the FUs obtained using the OEAT represent the enzymatic activity of human β-glucuronidase liberated from the fusion protein.

Histochemical Methods. β-Glucuronidase activity was determined on cryosections following the procedure described by Murray *et al.* (11). Controls were incubated with the histochemical substrate for β-glucuronidase dissolved in an excess of 10 mM D-saccharic-1,4-lactone monohydrate (saccharolactone; Aldrich) in 0.2 M acetate buffer, pH 5 (12).

Immunohistochemical Methods. Fusion protein present in tissues of mice or human tumor xenografts were detected using an alkaline phosphatase-labeled goat anti-human κ antibody or an alkaline phosphatase-labeled anti-human β-glucuronidase MAb as described (5).

Analysis of Prodrug and Drug in Plasma and Organs. After i.v. application of prodrug or drug, blood (200 μl) was collected by retroorbital punction (rats, mice) or by withdrawal from a venous catheter (monkeys) and was diluted with 50 μl 0.1 M citrate containing 10 mM saccharolactone. Plasma was prepared by centrifugation (10 min, 1000 × *g*) and further diluted (1:7) with phosphate H buffer, pH 6.0, containing 10 mM saccharolactone-0.01% BSA.

After weighing of organs and tumor tissues of nude mice 20 mM phosphate H buffer, pH 3.0, containing 10 mM saccharolactone-0.01% BSA was added (230 mg tissue/770 μl buffer). Tissues were homogenized by means of an Ultraturrax (1 min, 0°C). Homogenate (200 μl) was mixed with 40 μl silver nitrate (3.3%) and 160 μl acetonitrile, shaken (30 min), and centrifuged (12,000 × *g*, 5 min). Prior to HPLC analysis the supernatant (100 μl) was diluted with buffer, pH 6.0 (see above).

HPLC Analysis. The HPLC apparatus consisted of an autosampler (Abimed model 231), an automatic sample extraction system (AASP; Varian) equipped with minicartridges containing C_{18} reversed phase silica gel (Analytichem), a gradient pump (Gynkotek model 480), and a fluorescence detector (Shimazdu RF 535: excitation, 495 nm; emission, 560 nm). Before sample injection the minicartridges were preconditioned with 2.5 ml methanol and 1.5 ml 20 mM phosphate buffer, pH 6.0. After injection of the sample (350 μl) the cartridges were washed with 1.5 ml phosphate buffer, pH 6.0. Analytes retained on the reversed phase silica gel were then eluted by valve switching and connection of the minicartridges to the mobile phase. Chromatography was performed on reversed phase material (Nucleosil C_{18}, 5 μm particle size, 120 mm length, 4.5 mm inside diameter). Elution was done by a linear gradient composed of 2 components (A, 20 mM phosphate, pH 3.0; B, acetonitrile) with the following time-concentration profile: 0 min, 75% A, 25% B; 20 min, 25% A, 75% B; 30 min, 25% A, 75% B. Before starting the next run the column was allowed to equilibrate at starting conditions for 5 min.

Analysis of Therapeutic Activity. CD-1-*nu/nu* mice (7 animals/group) bearing s.c. growing LoVo colon carcinomas or Mz-Sto-1 stomach carcinomas received an i.v. bolus injection of 20 mg/kg of fusion protein on day −8. On day 0 these mice received an infusion of prodrug (250 mg/kg) over a period of 5 min. Other non-fusion protein-treated animals received on day 0 a 5-min infusion of physiological saline, prodrug (250 mg/kg), or doxorubicin (10 mg/kg) alone. Tumor growth was monitored over time by measurement of two perpendicular tumor diameters. Mean relative tumor areas were calculated from tumor diameters measured at individual days divided by tumor diameters measured at start of therapy (day 0). For treated groups the % T/C at individual days was calculated as

$$\%\ T/C = \frac{\text{Mean relative tumor area (treated group)}}{\text{Mean relative tumor area (control group)}} \times 100$$

Calculations. Time courses of prodrug concentrations resulting from i.v. injection in mice, rats, and monkeys were adjusted according to a biexponential function, corresponding to an open two compartment model with

$$C(t) = A \exp(-\alpha t) + B \exp(-\beta t)$$

$$\alpha = \ln(2)/t_{1/2,\alpha}$$

$$\beta = \ln(2)/t_{1/2,\beta}$$

The model equation was fitted to the data by means of nonlinear regression (PC HoeRep software) with weighed least squares and putting inverse quadratic weights on individual plasma concentrations.

K_m and V_{max} values were calculated using the computer program GraFit (Erithacus Software Ltd., Staines, United Kingdom).

RESULTS

Pharmacokinetics and Metabolism of Fusion Protein *in Vivo*

Quantitative Determination of Functionally Active Fusion Protein (OFAT). After a single i.v. injection of fusion protein with an immunoreactivity of >90% and a specific enzyme activity of 12 μmol/mg × min at pH 4.5 in tumor-bearing nude mice, a selective retention of functionally active fusion protein only was observed in CEA-expressing (Mz-Sto-1, LoVo) but not in CEA-negative (LXF-529) tumors (Table 1). At day 7 after i.v. injection of fusion protein, 200 or 400 ng of functionally active fusion protein were found per g of Mz-Sto-1 or LoVo tumors, respectively, whereas fusion protein concentration in LXF-529 tumors were below the detection limit of the assay (<1 ng/g). Thus, the *in vivo* specificity of fusion protein for CEA-expressing tumors is about 100 times higher than for not CEA-expressing tumors. At day 7 similar specificity ratios were also observed between fusion protein concentration in CEA-expressing tumors and plasma or normal tissues. Detectable but minor amounts of

Table 1 *In vivo distribution of enzymatically active fusion protein*

A. μg fusion protein localized/g organ (OFAT)

Time (h)	Tumor Mz-Sto-1	Plasma	Liver	Intestine	Kidney	Lung	Heart	Tumor LXF-529
0.05	3[a]	456	61	6	36	78	60	9
1.0	5	199	26	9	17	34	20	ND[b]
3.0	6	122	15	4	7	8	3	1
5.5	4	85	8	4	8	9	3	ND
24	5	19	2	0.6	3	2	0.5	2
168	0.2	<0.001	0.003	<0.001	<0.001	<0.001	<0.001	<0.001

B. μg fusion protein localized/g organ (OFAT)

Time (h)	Tumor LoVo	Plasma	Liver	Intestine	Kidney	Lung	Heart
0.05	3	387	61	4	24	51	25
3.0	5	134	18	7	8	12	11
24	7	12	2	1	1	4	1
72	3.0	0.6	0.07	0.02	0.04	0.2	0.05
168	0.4	<0.001	0.005	0.004	<0.001	0.003	<0.001

[a] Means of three organs per time (SD < ±50%).
[b] ND, not determined.

functionally active fusion protein were observed only in the liver, intestine, and lung.

The absolute masses of fusion protein detected in tumor and organs 3 min after i.v. injection sum up to approximately 100% of the injected dose, if the respective tissue weights of nude mice as well as the plasma content in the removed organs are considered. The absolute masses of fusion protein detected at later times are far below 100% and decrease continuously in the various organs.

Quantitative Determination of Functionally Active β-Glucuronidase (OEAT-OFAT). In addition to the analysis of functionally active fusion protein, tissues investigated in the above mentioned experiments were analyzed with respect to concentrations of non-fusion protein bound, functionally active human β-glucuronidase. In plasma of fusion protein-treated animals concentrations of free active β-glucuronidase were below 20 ng/ml during the observation period from 3 min to 168 h after fusion protein injection (Table 2), outlining the high stability of fusion protein in plasma. In contrast, high levels of free active β-glucuronidase were detected in the livers of these animals. In this tissue a time-dependent increase of free β-glucuronidase was observed with a maximum of 181–255 μg/g at 24 h after fusion protein application. Thereafter free β-glucuronidase values slowly declined to concentrations of 9–12 μg/g which were still present 168 h after fusion protein application. In opposition to the liver, concentrations of free human β-glucuronidase in other organs were found to be in the range of 1–14 μg/g and remained fairly constant during time.

Histochemical Determination of β-Glucuronidase. Histochemical analysis of normal tissues derived from either fusion protein-treated or untreated nude mice revealed that β-glucuronidase activity was exclusively associated with intracellular organelles. The microscopically visible red spots most probably are located in lysosomes of liver and other organs (Fig. 1). The semiquantitative histochemical procedure performed at different times shows that the intracellular β-glucuronidase activity is higher in liver parenchymal cells from nude mice treated with fusion protein than in untreated control mice in which the endogenous mouse β-glucuronidase activity is only marginally detectable. Addition of 10 mM saccharolactone, a selective competitive inhibitor of β-glucuronidases (12), completely abolishes the staining reaction (Fig. 1). As early as 3 min after fusion protein injection β-glucuronidase activity was detected in most liver parenchymal cells. Staining intensity remained constant up to 24 h and thereafter decreased to background levels until day 7.

Immunohistochemical Determination of Fusion Protein. In addition to the histochemical studies and the quantitative data generated using the OFAT/OEAT system, a semiquantitative immunohistochemical method was applied, allowing the visualization of the fusion protein on cryopreserved tumor tissue sections in its microenvironment. As early as 3 min after i.v. injection of 400 μg of fusion protein

Table 2 *In vivo distribution of enzymatically active free human β-glucuronidase*

A. μg free human β-glucuronidase localized/g organ (OEAT-OFAT)

Time (h)	Tumor Mz-Sto-1[a]	Plasma	Liver	Intestine	Kidney	Lung	Heart	Tumor LXF-529[a]
0.05	ND[b]	<0.02[c]	16	6	8	4	<0.02	ND
1.0	ND	<0.02	95	6	6	3	1	ND
3.0	ND	<0.02	105	6	4	3	1	ND
5.5	ND	<0.02	165	8	3	3	2	ND
24	ND	<0.02	255	10	4	4	3	ND
168	ND	<0.02	12	ND	ND	ND	ND	ND

B. μg free human β-glucuronidase localized/g organ (OEAT-OFAT)

Time (h)	Tumor LoVo[a]	Plasma	Liver	Intestine	Kidney	Lung	Heart
0.05	ND	<0.02	12	5	6	14	<0.02
3.0	ND	<0.02	135	5	4	6	0.5
24	ND	<0.02	181	6	4	2	4
72	ND	<0.02	157	5	1	1	2
168	ND	<0.02	9	5	3	2	3

[a] Not evaluable due to intracellular lysosomal human β-glucuronidase present in human tumor xenografts.
[b] ND, not determined.
[c] Mean of three organs per time (SD < ±50%).

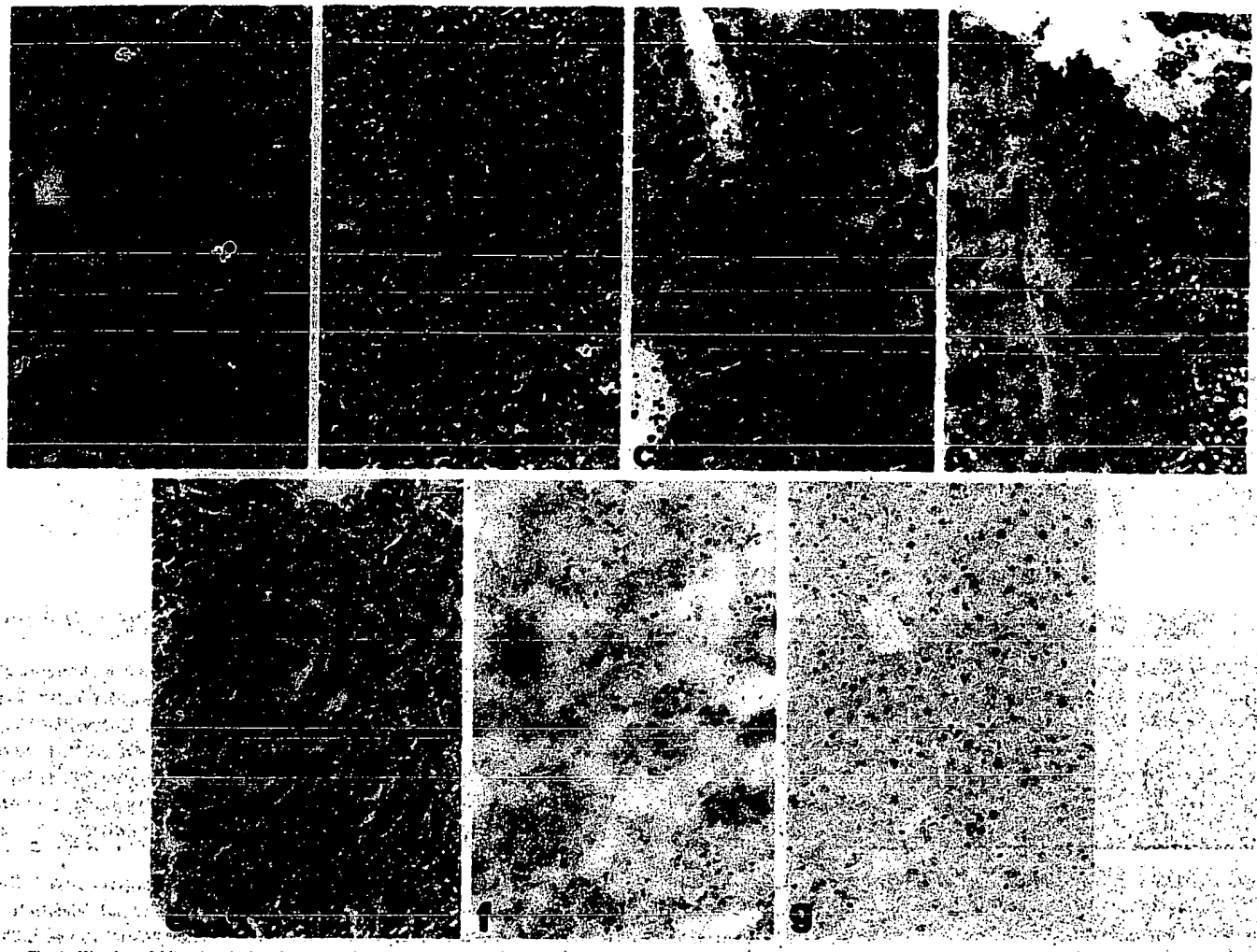

Fig. 1. Kinetics of histochemical staining reactions performed on cryopreserved mouse liver tissue sections derived from (*a*) untreated animals and (*b–f*) fusion protein (20 mg/kg)-treated animals. Livers removed 3 min (*b*), 3 h (*c*), 24 h (*d*), 72 h (*e*), 168 h (*f*) after fusion protein injection. (*g*) conditions as described for *a* but with addition of 10 mM saccharolactone (negative control). × 100.

in nude mice bearing established CEA-positive Mz-Sto-I xenografts a heterogeneous staining of certain areas in the tumor thin sections was seen (Fig. 2*c*). The strength of staining slightly increased up to 24 h after injection, however remaining heterogeneous (Fig. 2, *c–f*). Thereafter a decrease of staining intensity was observed which, however, at day 7 is above background level (compare Fig. 2, *a* and *b* with Fig. 2*g*).

In summary, with respect to normal organs, especially liver, the results from the quantitative analysis (OFAT, OEAT) as well as the histochemical data suggest that the kinetics of the fusion protein concentration in normal organs parallels its concentration in plasma. However, free human β-glucuronidase activity accumulates in liver parenchymal cells. This could be explained by a preferential uptake of the fusion protein into liver parenchymal cells and cleavage to an enzymatically active protein lacking the variable region. The concentrations of functionally active fusion protein in liver 7 days after its application are very low. The rapid uptake and degradation of the fusion protein, mainly in normal liver, are in contrast to its fate in the tumor in which 7 days after i.v. injection 200–400 ng of functionally active fusion protein can be detected per g tumor (Table 1). This obvious difference in the fate of the fusion protein in liver and normal organs compared to CEA-expressing malignant tissue results in the unexpectedly high specificity ratios reported above.

Furthermore, in contrast to the intracellular histochemical reactions in normal tissues, the immunohistochemical staining observed in tumor tissue was localized extracellularly. In addition, the semiquantitative immunohistochemical staining reactions are in agreement with the quantitative data presented above using the OFAT (Table 1). They clearly demonstrate that the fusion protein selectively binds to CEA-positive human tumor xenografts remaining there as a functionally active molecule for at least 7 days.

Plasma Stability of Prodrug and *in Vitro* Cleavage

In order to take advantage of the pharmacokinetics and metabolism of the fusion protein with respect to tumor chemotherapy a prodrug *N*-(4-β-glucuronyl-3-nitrobenzyloxycarbonyl)doxorubicin (Fig. 3) has been synthesized (6, 7). The prodrug is very stable under *in vitro* conditions in human, rat, or mouse plasma. After 50 h of incubation of the prodrug at 100 μg/ml maximally 20% of the prodrug is cleaved (Fig. 4). Addition of 1.6 μg/ml of fusion protein to a solution of prodrug (335 μg/ml) results in a quick disappearance of prodrug due to cleavage of the glucuronide moiety (Fig. 5). The resulting doxorubicin-spacer derivative spontaneously decomposes and gives raise to generation of doxorubicin (Fig. 5).

Based on a number of similar *in vitro* cleavage experiments in buffer at pH 7.2, K_m and V_{max} were calculated to be 1.3 mM and 0.635 nmol/min × μg at 37°C. Similar data have been reported previously

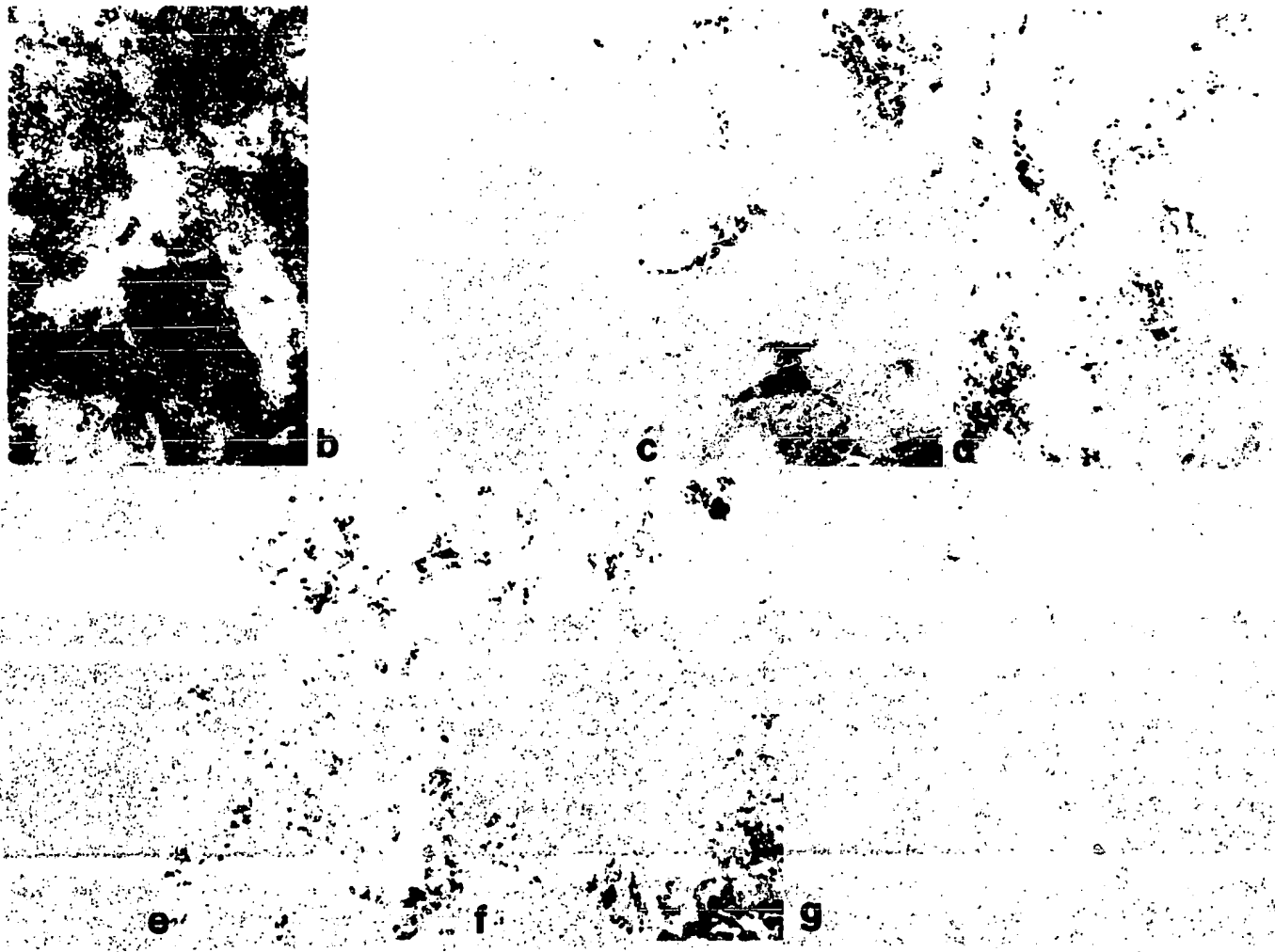

Fig. 2. Cryosections of Mz-Sto-1 xenografts stained with AP-labeled goat anti-human κ antibody (second antibody): (*a*) strong staining observed due to addition of 1 μg/ml fusion protein followed by second antibody (positive control); (*b*) no staining observed in untreated xenograft with second antibody (negative control); (*c–g*) heterogeneous staining observed 3 min, 3 h, 6 h, 24 h, and 168 h after i.v. application of 20 mg/kg fusion protein followed by detection with second antibody. × 100.

for other mammalian β-glucuronidases with respect to cleavage of the synthetic substrate 4-nitrophenyl-β-glucuronide (13).

Pharmacokinetics of Prodrug in Disease Free and Tumor-bearing Animals

The plasma half-life of the prodrug was determined after a single bolus i.v. injection of 50 mg/kg prodrug in tumor-free CD-1-*nu/nu* mice, in CD rats, and in *Macaca fascicularis* monkeys. Prodrug and drug concentrations were determined using reversed phase HPLC followed by data analysis with the HoeRep computer program.

In all three animal systems, pharmacokinetics of the prodrug fit into a two compartment model (Fig. 6) with an elimination half-life ($t_{1/2}\beta$) between 0.4 and 2.6 h and a distribution half-life ($t_{1/2}\alpha$) between 2 and 7 min (Table 3). Differences between the pharmacokinetic parameters are observed between the three species especially with respect to a significantly higher area under the curve value for monkeys compared to rodents (Table 3). For comparison distribution and elimination half-lives of doxorubicin are presented in rats.

After a 5-min infusion of prodrug in a therapeutic dosage (250 mg/kg) only minor amounts of doxorubicin were found in the plasma of tumor-free nude mice (Table 4). Depending on the evaluation time doxorubicin plasma concentration in these animals represents 0.08–3.2% of the corresponding plasma prodrug concentration. A similar

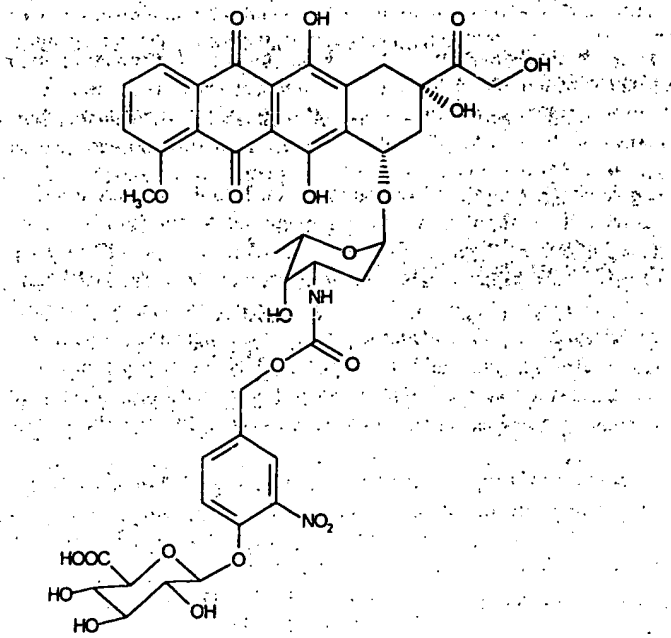

Fig. 3. Chemical structure of prodrug *N*-(4-β-glucuronyl-3-nitrobenzyloxycarbonyl)-doxorubicin.

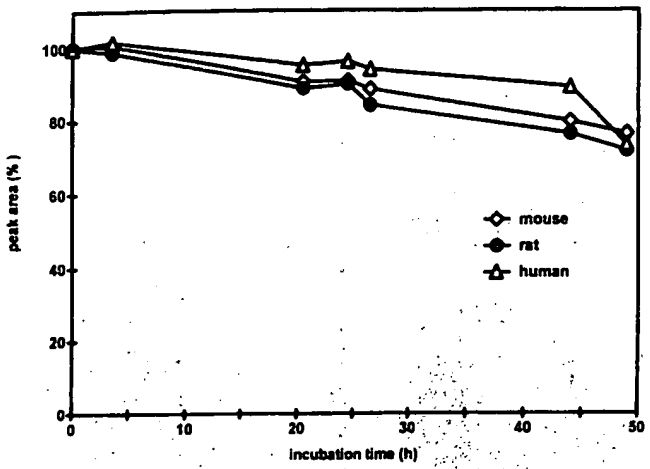

Fig. 4. Stability of prodrug in human, mouse, or rat plasma. Plasma containing 0.02 M citrate obtained from humans, mice, or rats was added to a solution of prodrug (200 μg/ml in 100 mM phosphate buffer, pH 7.35) (1:1) and incubated for various times at 37°C. Prodrug concentrations were analyzed according to the procedures described in "Materials and Methods." Data represent percentages of prodrug peak areas from the total peak area (value obtained at 0 min = 100%).

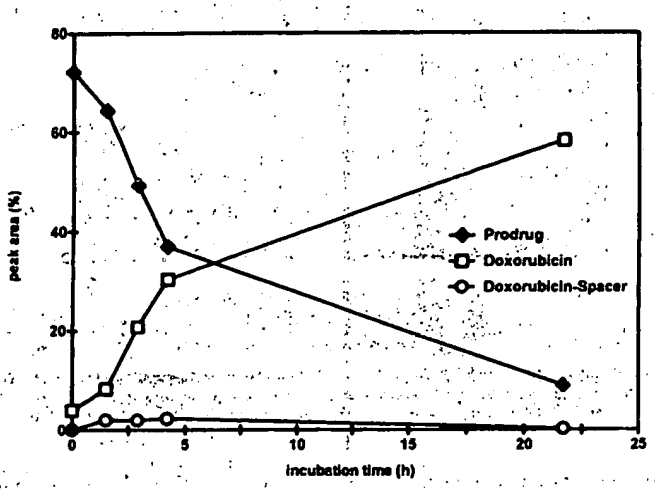

Fig. 5. Kinetics of cleavage of prodrug by fusion protein *in vitro*. Prodrug (335 μg/ml in 20 mM phosphate buffer, pH 7.2) was incubated with fusion protein (1.6 μg/ml) at 37°C. HPLC analysis of drug concentrations was performed according to the procedures described in "Materials and Methods."

percentage of liberation of doxorubicin from the prodrug (0.1–0.6%) was observed in *M. fascicularis* monkeys. Infusion of a maximum tolerated dose of free doxorubicin resulted in similar plasma concentrations as doxorubicin concentrations observed after prodrug infusion.

In a separate set of experiments fusion protein was injected into tumor (Mz-Sto-1)-bearing nude mice followed by an infusion of prodrug (250 mg/kg) 7 days later. At the same time other animals received an infusion of doxorubicin (12 mg/kg). The concentrations of doxorubicin in plasma of prodrug or doxorubicin-treated animals were found to be similar to those found in plasma of non-fusion protein-treated, tumor-free animals (Table 5). However, major differences were found with respect to doxorubicin concentrations in organ and tumor tissues. In mice treated with fusion protein and prodrug the amounts of doxorubicin in various organ tissues were found up to 5-fold lower than the amounts observed in doxorubicin-treated animals. In contrast, the amount of drug present in tumor tissues was 4–12-fold higher for fusion protein- and prodrug-treated mice than in animals which received a single application of doxorubicin. Thus, these data show that treatment of tumor-bearing mice with fusion protein and prodrug results in an increase of drug concentration in the target tissue (Mz-Sto-1 tumor) and a reduction of drug concentration in nontarget tissues.

MTD of Prodrug and Drug *in Vivo*

The MTD of prodrug and drug was determined by 5-min i.v. infusions of increasing dosages of prodrug or drug in CD-1-*nu/nu* mice. MTD of the drug was 12 mg/kg (0.022 mmol/kg), and that of the prodrug was >1600 mg/kg (1.8 mmol/kg), if applied in doses of 800 mg/kg in 6-h intervals. Applications of a single dose larger than 800 mg/kg were not possible due to the water solubility of the prodrug (30 mg/ml). According to these data, the prodrug is at least 130-fold less toxic (w/w) *in vivo* than the drug. MTD of prodrug, if applied 8 days after fusion protein application, was >1600 mg/kg as well.

Therapeutic Efficacy

Based on the *in vivo* distribution and tumor retention data generated using the fusion protein as well as on the pharmacokinetics and *in vivo* prodrug activation data obtained, therapy experiments were performed. Seven days after injection of 20 mg/kg of fusion protein into nude mice bearing established CEA-expressing human tumor xenografts (tumor diameter, 3–5 mm) (LoVo) 250 mg/kg of prodrug were infused. In addition, separate groups of animals were treated with prodrug alone, doxorubicin, or physiological saline.

Therapeutic efficacy was documented by monitoring tumor growth (Fig. 7). Significant growth retardation with partial regression was obtained only in those animals receiving fusion protein and prodrug (26% T/C, day 24). Prodrug alone (68% T/C) or doxorubicin (61% T/C), respectively (both on day 24) had no significant antitumoral effect against this particular tumor. These superior therapeutic effects were obtained, without any obvious signs of toxicity to the animals, at ≈1/6 of the MTD of the prodrug.

DISCUSSION

This preclinical study demonstrates that appropriate *in vivo* application of a tumor-selective humanized fusion protein and a nontoxic prodrug generate tumor therapeutic effects superior to those with conventional chemotherapy (Fig. 7). These therapeutic effects were obtained in a human tumor xenograft model relatively resistant to

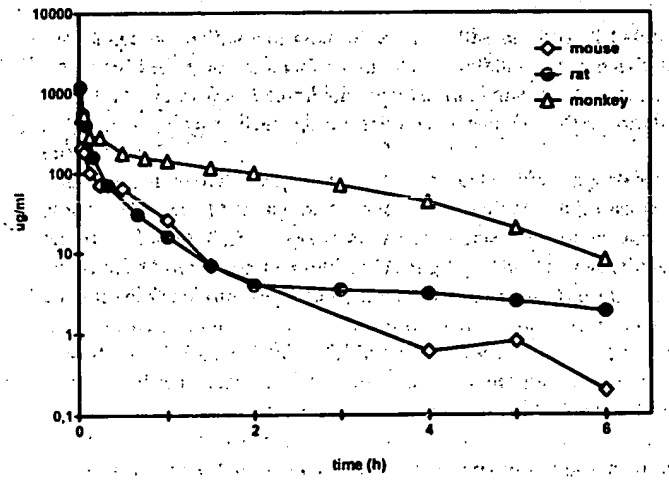

Fig. 6. Plasma pharmacokinetics of prodrug in 3 species. Fifty mg/kg of prodrug were injected i.v. At the indicated times, blood samples were taken from sacrificed mice and rats or via cannulation (monkeys). Prodrug concentrations in plasma were determined using reversed phase HPLC as described in "Materials and Methods."

Table 3 *Pharmacokinetic parameters of prodrug and doxorubicin in different species*

Compound	Species	Strain	$t_{1/2}\alpha$ (min)	$t_{1/2}\beta$ (h)	A (μg/ml)	B (μg/ml)	AUC[a] (μg × h/ml)
Prodrug	Mouse	CD 1-*nu/nu*	2	0.4	198	115	82
Prodrug	Rat	CD	7	2.6	532	8.8	127
Prodrug	Monkey	M. fascicularis	3	1.3	455	261	522
Doxorubicin	Rat	CD	2	8.1	23	0.2	3

[a] AUC, area under the concentration/time curve.

Table 4 *Pharmacokinetics of prodrug and doxorubicin in untreated nude mice*

Time (min)	Prodrug (250 mg/kg)		Doxorubicin (12 mg/kg)
	Prodrug (μg/ml)	Doxorubicin (μg/ml)	Doxorubicin (μg/ml)
1	4408[a]	4	5
5	2212	2	0.8
10	1632	3	0.7
30	492	2	0.2
60	87	1.0	0.2
90	6.0	0.2	0.17
1440	0.8	0.02	0.02

[a] Mean of three organs per time (SD < ±50%).

doxorubicin treatment and with a single cycle of fusion protein and prodrug. Due to the presumably low immunogenicity of the fusion protein consisting of a humanized CEA-specific binding region and human β-glucuronidase (5), repetitive applications of fusion protein should be possible. Furthermore, the prodrug dose used in our therapy experiment (250 mg/kg) is only approximately one-sixth of the MTD of the prodrug. Therefore, repetitive treatment cycles probably can be applied in patients, hopefully resulting in even superior therapeutic effects than those reported in our *in vivo* nude mouse studies.

In contrast, the application of repetitive treatment cycles does not seem to be possible without immunosuppressive therapy in systems containing mouse MAbs chemically linked to xenogenic enzymes (14). In addition, to be effective some xenogenic antibody-enzyme conjugate systems (3) need as a third component the injection of a galactosylated anti-enzyme MAb to clear the xenogenic antibody-enzyme conjugate from the plasma before prodrug injection. Such a clearing step is not needed in our system, because the fusion protein is quickly eliminated from plasma probably by internalization into parenchymal cells of the liver (Fig. 1). Internalization is most probably mediated by mannose 6-phosphate- and galactose receptor-mediated uptake known to be a highly active internalization pathway in liver parenchymal cells. Galactosylated and mannosylated glycoproteins like human β-glucuronidase are efficiently taken up and transported to the lysosomal compartment, where enzymatic degradation occurs as observed similarly for the fusion protein.[3] Intracellularly, the fusion protein is cleaved to enzymatically active human β-glucuronidase lacking the CEA-binding region (Table 2). During time, intracellularly accumulated human β-glucuronidase activity is slowly reduced. This efficient internalization and degradation mechanism is one of the parameters responsible for the very high ratios (>100:1) of functionally active fusion protein between tumor and plasma or organs obtained at day 7 after fusion protein injection as shown in two independent kinetic studies using two different CEA-expressing human tumor xenografts (Table 1).

The mechanistic interpretation suggested above is supported by the high stability of the fusion protein (>80%) during incubation for 4 weeks at 37°C in rodent or human plasma arguing against easily accessible plasmatic protease cleavage sites (data not shown). This finding which is in concordance with the nondetectable amounts of free human β-glucuronidase in plasma after fusion protein application (Table 2) supports the hypothesis that the increase of free β-glucuronidase in liver parenchymal cells is rather due to internalization of the fusion protein into the liver cells and intracellular cleavage of the fusion protein than to extracellular removal of the V-region followed by internalization of functionally active β-glucuronidase.

Despite of its high molecular weight (>250,000) under nondenaturing conditions and the known diffusion barriers reported for solid tumors (15), the fusion protein is able to penetrate human tumor xenograft tissues (Fig. 2). Although the staining reaction after i.v. application of the fusion protein remains heterogeneous (Fig. 2), the amounts of functionally active fusion protein in the tumor at day 7 (Table 1) are still suitable to activate the prodrug *in vivo* (Table 5). Compared to standard chemotherapy using doxorubicin, the doxorubicin concentrations generated by fusion protein-mediated prodrug activation results in 4–12-fold higher doxorubicin concentrations in the tumor and up to 5-fold lower doxorubicin concentrations in normal tissues (Table 5). The pharmacokinetic advantage observed

Table 5 *Plasma pharmacokinetics and tissue distribution of prodrug and doxorubicin in fusion protein-treated tumor-bearing nude mice*

Tissue	Time (h)	Fusion protein (20 mg/kg) + prodrug (250 mg/kg)		Doxorubicin (12 mg/kg)
		Prodrug (μg/ml)	Doxorubicin (μg/ml)	Doxorubicin (μg/ml)
Plasma	0.03	1040[a]	2.1	7.2
	0.08	928	2.2	0.7
	0.17	508	1.2	0.5
	0.5	321	1.8	0.3
	1.0	128	1.3	0.3
	4.0	2.7	0.03	0.1
	8.0	0.9	0.03	0.07
Tumor (Mz-Sto-1)	0.5	67	5.7	1.4
	1.0	45	10	2.2
	4.0	11	16	1.8
	8.0	5.0	17	1.4
Liver	0.5	88	11	23
	1.0	86	13	23
	4.0	2.2	5.8	17
	8.0	2.3	5.0	9.7
Lung	0.5	166	6.5	12
	1.0	14	5.2	16
	4.0	1.0	4.8	22
	8.0	0.8	6.8	15
Kidney	0.5	4071	20	57
	1.0	23	15	40
	4.0	2.8	11	30
	8.0	0.01	13	15
Spleen	0.5	52	5.2	19
	1.0	4.7	6.1	4.5
	4.0	1.9	8.0	4.8
	8.0	0.1	2.1	3.9
Muscle	0.5	38	1.9	3.5
	1.0	1.9	2.1	9.6
	4.0	1.0	2.3	4.7
	8.0	0.03	2.2	5.2
Heart	0.5	83	4.0	9.1
	1.0	5.1	4.5	11
	4.0	0.3	3.3	14
	8.0	0.01	2.1	5.8

[a] Mean of three organs per time (SD < ±50%).

[3] Manuscript in preparation.

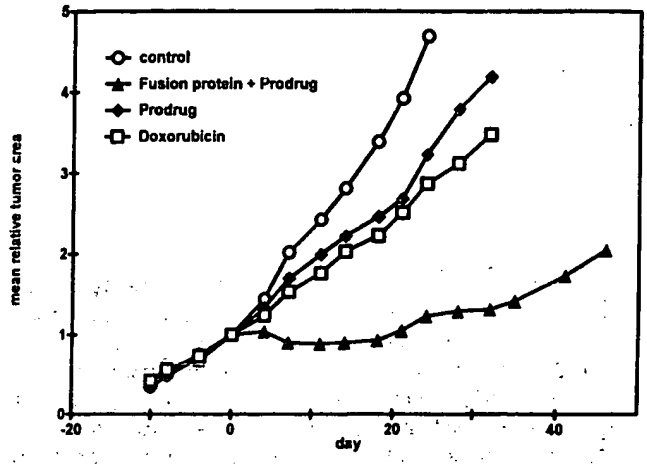

Fig. 7. Therapeutic efficacy of the combined application of fusion protein and prodrug in nude mice bearing s.c. growing LoVo colon carcinomas. Tumor-bearing nude mice were treated with fusion protein (20 mg/kg, i.v. bolus) on day −8 followed by a 5-min infusion of prodrug (250 mg/kg) on day 0 at which time the mean tumor area was about 35 mm^2. Other groups received 5-min infusions of physiological saline, prodrug (250 mg/kg), or doxorubicin (10 mg/kg) alone.

using the FMPA system can be explained by the high hydrophilicity of the prodrug resulting preferentially in doxorubicin liberation mainly at sites where extracellularly accessible β-glucuronidase is available. However, small amounts of doxorubicin are probably generated from the prodrug in plasma and normal tissues catalyzed by the minute amounts of accessible plasmatic and normal tissue-associated glucuronidase. In contrast, the unfavorable normal tissue doxorubicin levels obtained after i.v. injection of doxorubicin are a consequence of the high lipophilicity of the drug leading to rapid tissue uptake and a concomitant decrease of plasma levels. Furthermore, the advantageous *in vivo* drug distribution using FMPA is only possible, because the prodrug has several favorable characteristics: (*a*) the high *in vivo* tolerability (>1600 mg/kg) of the prodrug should be emphasized resulting in an exceptionally high detoxification factor between prodrug and drug (>130-fold); (*b*) it has long-term plasma stability in mouse, rat, and human plasma (Fig. 4) avoiding significant unspecific drug liberation; (*c*) the *in vitro* prodrug activation kinetics performed in plasma show that addition of enzymatically active fusion protein leads to a rapid cleavage of the prodrug to the drug (Fig. 5), with similar characteristics as observed for the activation of conventional substrates such as methylumbelliferyl-β-glucuronide or 4-nitrophenyl-β-glucuronide used to evaluate natural human β-glucuronidase. In contrast to the ADEPT system suggested by the groups of Senter (16), Meyer (17), and Bagshawe (18) using xenogenic enzymes like β-lactamase or carboxypeptidase G2 as catalytic moiety, our FMPA system uses human β-glucuronidase as a catalytic moiety. The turnover rate of our fusion protein is significantly below the activities reported for the above mentioned antibody-enzyme conjugates due to the high K_m (1.3 mM) and relatively low V_{max} (0.635 nmol/min × μg, pH 7.2) of human β-glucuronidase. This disadvantage of our fusion protein is more than compensated by its superior pharmacokinetics resulting in unusually high specificity ratios totally in agreement with the pharmacodynamic model constructed by Yuan *et al.* (19). These authors suggest that in two step systems a significant delay between the first injection of the catalytic component and the prodrug will result in little toxicity and superior therapeutic efficacy.

Indeed, after a delay of 7 days between fusion protein injection and prodrug infusion with a single dose of 250 mg/kg of prodrug, therapeutic effects in a CEA-positive human tumor xenograft system were observed which are superior to treatment with standard doxorubicin therapy (Fig. 7). This superior efficacy of FMPA is supported by the more favorable *in vivo* drug distribution in the tumor as well as in normal tissues (Table 5) as discussed above. A comparison of our *in vivo* drug distribution data after FMPA with those obtained in the ADEPT system (18) reveals that in our system a tumor-selective drug deposition is observed, whereas the ADEPT system based on antibody-carboxypeptidase G2 conjugates leads to a more systemic drug distribution. The advantageous drug distribution in our system is mediated not only by the high *in vivo* specificity ratio of the fusion protein but also by the high prodrug stability in plasma and its favorable pharmacokinetic characteristics (Table 3). Thus, the finding that plasma area under the curve values were significantly higher in monkeys than in rodents (Table 3) suggests that the prodrug dose needed in human beings can presumably be reduced by a factor of 5–10. This hypothesis is based on the assumption that tumor tissue prodrug levels are influenced by the respective prodrug plasma levels and that tumor tissue prodrug levels allowing efficient catalysis are available for prolonged periods of time in monkeys and probably also in human beings compared to rodents.

In summary, the prospective low immunogenicity of our fusion protein and the high specificity ratio obtained *in vivo* combined with tumor-selective disposition of doxorubicin after prodrug activation resulting in superior therapeutic effects recommend our FMPA system for clinical development.

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REFERENCES

1. Ford, J. M., and Hait, W. N. Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol. Rev., *42:* 155–199, 1990.
2. Senter, P. D., Wallace, P. M., Svensson, H. P., Vrudhula, V. M., Kerr, D. E., Hellström, I., and Hellström, K. E. Generation of cytotoxic agents by targeted enzymes (review). Bioconj. Chem., *4:* 3–9, 1993.
3. Sharma, S. K., Bagshawe, K. D., Springer, C. J., Burke, P. J., Rogers, G. T., Boden, J. A., Antoniw, P., Melton, R. G., and Sherwood, R. F. Antibody directed enzyme prodrug therapy (ADEPT): a three phase system. Dis. Markers, *9:* 225–231, 1991.
4. Bagshawe, K. D., Sharma, S. K., Springer, C. J., Antoniw, P., Boden, J. A., Rogers, G. T., Burke, P., and Melton, R. G. Antibody directed enzyme prodrug therapy (ADEPT): clinical report. Dis. Markers, *9:* 233–238, 1991.
5. Bosslet, K., Czech, J., Lorenz, P., Sedlacek, H. H., Schuermann, M., and Seemann, G. Molecular and functional characterization of a fusion protein suited for tumor specific prodrug activation. Br. J. Cancer, *65:* 234–238, 1992.
6. Jacquesy, J.-C., Gesson, J.-P., Monneret, C., Mondon, M., Renoux, B., Florent, J.-C., Koch, M., Tillequin, F., Sedlacek, H. H., Kular, C., and Gaudel, G. Prodrogues glycosylées, leur procédé de préparation et leurs utilisation. Demande de Brevet Européen. EP 0 511 917 A1.
7. Anadrianomenjanahary, S., Koch, M., Tillequin, F., Michel, S., Monneret, C., Florent, J. C., Gesson, J. P., Jacquesy, J. C., Mondon, M., and Bosslet, K. *N*-(D-Glycopyranosyl)-chlorbenzyloxycarbonyl daunorubicine prodrugs and their enzymatic cleavage. Proceedings of the International Carbohydrate Symposium Paris, *A 264:* 299, 1992.
8. Bosslet, K., Steinsträsser, A., Schwarz, A., Harthus, H. P., Lüben, G., Kuhlmann, L., and Sedlacek, H. H. Quantitative considerations supporting the irrelevance of circulating serum CEA for the immunoscintigraphic visualisation of CEA expressing carcinomas. Eur. J. Nucl. Med., *14:* 523–528, 1988.
9. Bruyńck, A., Seemann, G., and Bosslet, K. Characterization of a humanized bispecific monoclonal antibody for cancer therapy. Br. J. Cancer, *67:* 436–440, 1993.
10. Kyle, J. W., Galvin, N., Vogler, C., and Grubb, J. H. β-Glucuronidase (GUS) Assay in Animal Tissue. GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression, pp. 189–203. New York: Academic Press, 1992.
11. Murray, G. I., Burke, M. D., and Ewen, S. W. B. Enzyme histochemistry on freeze-dried resin-embedded tissue. J. Histochem. Cytochem., *37:* 643–652, 1989.
12. Lojda, Z., Gossrau, R., and Schiebler, T. H. Enzyme Histochemistry, pp. 166–167. Berlin: Springer Verlag, 1979.
13. Tomino, S., Paigen, K., Tulsiani, T. P. P., and Touster, O. Purification and chemical properties of mouse liver lysosomal β-glucuronidase. J. Biol. Chem., *250:* 8503–8509, 1975.
14. Bagshawe, K. D., Sharma, S. K., Springer, C. J., Antoniw, P., Rogers, G. T., Burke, P. J., Melton, R., and Sherwood, R. Antibody-enzyme conjugates can generate cytotoxic drugs from inactive precursors at tumor sites. Antibody Immunoconj.

Radiopharm., *4:* 915–922, 1991.
15. Jain, R. K. Transport of molecules in the tumor interstitium: a review. Cancer Res., *47:* 3039–3051, 1987.
16. Goshorn, S. C., Svensson, H. P., Kerr, D. E., Sommerville, J. E., Senter, P. D., and Fell, H. P. Genetic construction, expression and characterization of a single chain anti-carcinoma antibody fused to β-lactamase. Cancer Res., *53:* 2123–2127, 1993.
17. Meyer, D. L., Jungheim, L. N., Law, K. L., Mikolajczyk, S. D., Shepherd, T. A., Mackensen, D. G., Briggs, S. L., Starling, J. J. Site-specific prodrug activation by antibody-β-lactamase conjugates: regression and long-term growth inhibition of human colon carcinoma xenograft models. Cancer Res., *53:* 3956–3963, 1993.
18. Antoniw, P., Springer, C. J., Bagshawe, K. D., Searle, F., Melton, R. G., Rogers, G. T., Burke, P. J., and Sherwood, R. F. Disposition of the prodrug 4-(bis(2-chloroethyl)amino)benzoyl-L-glutamic acid and its active parent drug in mice. Br. J. Cancer, *62:* 909–914, 1990.
19. Yuan, F., Baxter, L. T., and Jain, R. K. Pharmacokinetic analysis of two-step approaches using bifunctional and enzyme-conjugated antibodies. Cancer Res., *51:* 3119–3130, 1991.